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Identification and expression profile of multiple genes in the anterior kidney of channel catfish induced by modified live *Edwardsiella ictaluri* vaccination

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ABSTRACT

Using PCR-select subtractive cDNA hybridization technique, 57 expressed sequence tags (ESTs) were isolated from 240 clones of a modified live *Edwardsiella ictaluri* vaccinated *vs.* sham-vaccinated channel catfish anterior kidney subtractive library. The transcription levels of the 57 ESTs in response to *E. ictaluri* vaccination were then evaluated by quantitative PCR (QPCR). Of the 57 ESTs, 43 were induced at least 2-fold higher in all three vaccinated fish compared to unvaccinated control fish. Of the 43 upregulated genes, five were consistently upregulated greater than 10-fold, including two highly upregulated (>20-fold) glycosyltransferase and Toll-like receptor 5. The transcriptional levels of GTPase 1, coatomer protein complex zeta 1, and type II arginine deiminase were consistently induced greater than 10-fold. MHC class I α chain and transposase were upregulated greater than 10-fold in two of the three vaccinated fish. The 43 upregulated genes also included 19 moderately upregulated (3–10-fold) and 17 slightly upregulated (2–3-fold). Our results suggest that subtractive cDNA hybridization and QPCR are powerful cost-effective techniques to identify differentially expressed genes in response to modified live *E. ictaluri* vaccination.

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1. Introduction

Enteric septicemia of catfish (ESC), the most prevalent disease affecting farm-raised channel catfish, *Ictalurus punctatus*, is caused by *Edwardsiella ictaluri*, a facultative intracellular Gram-negative flagellated bacterium akin to phylogenetically related *Salmonella* (Thune et al., 1997; Zhang and Arias, 2007). ESC is generally an acute septicemia that develops very quickly, especially in the temperature range of 22–28 °C. Signs of the disease have been observed within 2 days after immersion challenge and heavy mortalities have been reported as early as 4 days after infection (Newton et al., 1989; Wolters and Johnson, 1994; Thune et al., 1997).

To control ESC, live attenuated *E. ictaluri* vaccines have been developed to protect catfish (Wise et al., 2000; Shoemaker et al., 1999, 2002, 2007; Karsi et al., 2009). Several studies have demonstrated that protective immunity in channel catfish against *E. ictaluri* is largely mediated by cellular immune responses with humoral antibodies having a secondary function (Ellis, 1999; Shoemaker and Klesius, 1997). Like *Salmonella*, *E. ictaluri* can survive and replicate intracellularly (Steele-Mortimer et al., 2000; Skirpstunas and Baldwin, 2002; Thune et al., 2007; Russo et al., 2009), further suggesting that the cellular immune response plays an important role in combating ESC.

The innate immune system is the first line of host defense against pathogens and plays a vital role in maintaining host–microbe homeostasis (Bingle and Craven, 2004). Bony fish have very quick and powerful defense mechanisms to a wide range of pathogens (Bayne et al., 2001; Ellis, 2001). The host immune system recognizes invading pathogens by their

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highly conserved pathogen-associated molecular patterns (PAMPs), which are unique to these pathogens and are normally not shared by host cells. Recognition mediated by pattern recognition receptors (PRRs) will then initiate the inflammatory processes (Medzhitov and Janeway, 2002; Magor and Magor, 2001; Hoffman et al., 1999). Many components of PRRs, such as Toll-like receptors (TLRs), are evolutionarily conserved from insects to humans (Kimbrell and Beutler, 2001). TLRs function in innate immunity through recognizing the conserved pathogen-associated molecular patterns (PAMPs) of an invading pathogen and eliciting inflammatory and immune responses (Medzhitov and Janeway, 2000). To date, 13 TLRs (TLR1–TLR13) have been identified in mammals and five TLRs (TLR2, TLR3, TLR5, TLR20, and TLR21) have been reported in channel catfish (Bilodeau and Waldbieser, 2005; Baoprasertkul et al., 2007a,b). The best characterized ligands that TLRs recognize include: (1) lipoproteins by TLR2; (2) dsRNA by TLR3; (3) lipopolysaccharide (LPS) by TLR4; and (4) flagellin by TLR5.

To understand the molecular mechanism involved in host immune response to *E. ictaluri* infection in channel catfish, expression profiles of different genes have been selected and studied. For example, the expression of channel catfish TLR3 and TLR5 in response to *E. ictaluri* challenge through immersion has been studied at 2, 5, 8, and 21 days post-challenge and it has been demonstrated that the expression of TLR3 is significantly upregulated in the head kidney of catfish 2 days after immersion challenge (Bilodeau and Waldbieser, 2005). The expression of channel catfish TLR2 in response to *E. ictaluri* challenge through immersion has been studied at 4, 24, and 72 h post-challenge and it has been demonstrated that TLR2 is downregulated at all time points in the head kidney after immersion challenge (Baoprasertkul et al., 2007a), suggesting that anterior kidney play an important role in the hose immune defense system. A recent study has demonstrated that modified live vaccinated catfish are able to control the dispersion of *E. ictaluri* in the anterior kidney (Russo et al., 2009), further suggesting that anterior kidney plays an important role in the immune defense system.

In addition to TLRs, many other genes such as chemokines, antimicrobial peptides, and pro-inflammatory cytokines have been reported to be upregulated in channel catfish challenged by *E. ictaluri* (Peatman et al., 2005, 2006; Chen et al., 2005; Baoprasertkul et al., 2004; Bao et al., 2006; Yeh and Klesius, 2007, 2008). To understand the transcriptional regulation of genes in response to modified live *E. ictaluri* vaccination in the anterior kidney without any preconception of their identities, we used PCR-select suppression subtractive hybridization in this study. Since its first introduction to researchers (Diatchenko et al., 1996), suppression subtractive hybridization has been widely used by researchers in fish innate immunology (Dios et al., 2007; Zhang et al., 2007a,b) and other fields of science (Singh et al., 2008; Zhao et al., 2008; Zhou et al., 2008; Pridgeon et al., 2009) because this technique does not require any previously known genome information for the organism (Sternberg and Gepstein, 2007; Hillmann et al., 2009). We used quantitative PCR (QPCR) to compare the transcriptional levels of genes in vaccinated fish and unvaccinated fish because QPCR has tremendous sensitivity and requires little or no post-amplification processing (Wong and Medrano, 2005). Furthermore, QPCR is a highly reproducible technique for the gel-free detection and quantification of mRNA (Ashton and Headrick, 2007). Using PCR-select subtractive cDNA hybridization technique, we identified 57 different genes from 240 clones of modified live *E. ictaluri* vaccinated *vs.* non-vaccinated channel catfish anterior kidney subtractive library. The transcriptional profiles of the 57 genes in response to *E. ictaluri* vaccination and their putative functions in immune defense were discussed in this study.

2. Materials and methods

2.1. Experimental fish, vaccine strain, and vaccination protocol

Channel catfish fry (NWAC-103 strain) were obtained from the USDA-ARS Catfish Genetic Research Unit, Stoneville, MS and maintained at the USDA-ARS-Aquatic Animal Health Unit at Auburn, AL. Fish were maintained in dechlorinated city water in 340 L tanks to ensure that the catfish fingerlings remained naïve to *E. ictaluri* during grow-out. Catfish fingerlings were grown for 8 months before vaccination and were 186 ± 9 g at time of vaccination. Prior to vaccination fish were moved to 208-L flow-through aquaria and acclimated for 14 days. Fish were vaccinated with AQUAVAC-ESC^{TM} following the established protocol from the manufacturer (Intervet/Schering Plough, Millsboro, DE). The vaccine was based on the modified RE-33 *E. ictaluri* developed by Klesius and Shoemaker (1999). Briefly, the vaccine was thawed at 26 °C in a water bath prior to dilution in 18.9 L of water at 26 ± 2 °C. Three channel catfish (*I. punctatus*) were transferred into one half (9.45 L) of the prepared vaccine bath provided with aeration through air stones via an air blower and held for 2 min. After the 2 min exposure time, an equal volume of water was added and the fish were allowed to remain in the vaccine bath for 15 min prior to release into 208-L flow-through tanks with constant aeration at water temperature of 26 ± 2° C and a 12:12 h light:dark photoperiod. The bacterial concentration was 6.5×10^6 CFU/mL in the vaccine bath. Three additional catfish were sham-vaccinated in immersion water only following the same procedure as above but without the addition of vaccine. All fish were returned to aquarium after vaccination or sham vaccination. At 48 h post-vaccination, fish were euthanized with MS-222 (300 mg/L). The anterior kidney tissue from each fish was collected and flash frozen in liquid nitrogen during collection. All samples were stored at −80 °C until RNA extraction.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was isolated from anterior kidney tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. All total RNAs were quantified on a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE). Total RNAs were resuspended in distilled water and stored at −80 °C. The first strand cDNAs used for quantitative PCR were synthesized using AMV reverse transcriptase (Invitrogen,

Carlsbad, CA). For subtractive library construction, total RNA were pooled from the three vaccinated or non-vaccinated fish samples. cDNAs were then synthesized from the pooled total RNAs using PCR-selected cDNA Subtraction Kit (Clontech, Palo Alto, CA) as described by the manufacturer. The cDNAs that contain specific transcripts are referred to as "testers" (i.e. from vaccinated fish) and the reference cDNAs are referred to as "drivers" (i.e. from non-vaccinated fish). The double-stranded cDNAs of both testers and drivers were digested with RsaI to create smaller blunt-ended fragments to be used as testers or drivers according to the manufacturer's instruction (Clontech, Palo Alto, CA). The tester cDNAs were then subdivided into two portions (A and B) and modified by ligating with cDNA adaptors 1 and 2 (provided by the kit), respectively.

2.3. Construction of subtractive cDNA library

Two-step subtractive hybridizations were performed according to procedures used previously (Pridgeon and Liu, 2003). Briefly, in the first step hybridization, two primary hybridization reactions (A and B) were formed by adding excess amounts of unmodified driver cDNA to separate portions A and B of tester cDNA samples at a 50:1 ratio. The samples were denatured for 2 min at 98 °C and allowed to anneal for 8 h at 68 °C. The remaining single-stranded, adaptor-ligated tester cDNAs were dramatically enriched in each hybridization reaction for overexpressed sequences because non-target cDNAs present in the tester and driver formed hybrids. For the second step hybridization, A and B primary hybridization reaction solutions were mixed together without denaturing. These new hybrids were double-stranded tester molecules with different 5′-ends corresponding to the sequences of two different adaptors. Freshly denatured driver DNA was added to the reaction without denaturing the subtraction mix to further enrich new double-stranded tester molecules that are differentially expressed. After filling in the adapter ends with DNA polymerase, overexpressed sequences (tester cDNA) had different annealing sites on their 3′- and 5′-ends. The molecules were then subjected to suppression subtraction PCR as described by the manufacturer (Clontech, Palo Alto, CA). The PCR products were then cloned into pGEM-T easy vector (Promega, Madison, WI). Plasmids were then transformed into One Shot® TOP10 competent cells (Invitrogen, Carlsbad, CA). Transformed cells were then plated out on Luria-Bertani (LB) plates containing ampicillin (100 μg/mL) and X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) (40 μg/mL).

2.4. PCR analysis of subtraction efficiency

To evaluate the subtraction efficiency, the relative amount of the constitutively expressed reference gene 18S rRNA was compared in subtracted cDNA and unsubtracted cDNA after 15, 20, 25, 30, 35, and 40 cycles of PCR. Primers used for the amplification of the 18S rRNA gene was 18S-F (5′-ATGGCCGTTCTTAGTTGGTG-3′) and 18S-R (5′-TAGGTAGCACACGCTGATCG-3′). The two primers were designed based on channel catfish 18S small subunit ribosomal RNA gene sequence (GenBank accession no. BE469353).

2.5. Plasmid DNA isolation and sequencing

From the library, a total of 240 white colonies were present and subsequently picked to grow overnight in LB broth in the presence of ampicillin (100 μg/mL) in the Innova™ 4000 Incubator Shaker (New Brunswick Scientific, Edison, NJ) at 37 °C and 235 rpm settings, respectively. Plasmid DNAs were isolated with QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA). Plasmid DNAs were then digested by *EcoRI* at 37 °C for 1 h and subjected to 1% agarose gel electrophoresis. Plasmid DNAs that contained inserts were then sent to USDA-ARS Mid South Genomic Laboratories in Stoneville, MS for sequencing with an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were then analyzed using the National Center for Biotechnology Information (NCBI) BLAST program to search for sequence homologies.

2.6. Primer design and quantitative PCR

Sequencing results of different clones were used to design gene-specific primers by using Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). QPCR was performed on an Applied Biosystems 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). For each cDNA sample, channel catfish 18S ribosomal RNA primers were included as an internal control to normalize the variation of cDNA amount. All QPCR was performed on an Applied Biosystems 7000 Real-Time PCR System (ABI, Foster City, CA) using Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen, Carlsbad, CA) in a total volume of 12.5 μL. The QPCR mixture consisted of 1 μL of cDNA, 0.5 μL of 5 μM gene-specific forward primer, 0.5 μL of 5 μM gene-specific reverse primer and 10.5 μL of 1× SYBR Green SuperMix. The QPCR thermal cycling parameters were 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All QPCR was run in duplicate for each cDNA sample and three fish cDNA samples were analyzed by QPCR.

2.7. QPCR data analysis

The relative transcriptional levels of different genes were determined by subtracting the cycle threshold (C_t) of the sample by that of the 18S rRNA, the calibrator or internal control, as per the formula: $\Delta C_t = C_t$ (sample) $- C_t$ (calibrator). The relative expression level of the specific gene in *E. ictaluri* RE-33 vaccinated fish compared to that in non-vaccinated fish was then calculated by the formula $2^{\Delta\Delta C_t}$ where $\Delta\Delta C_t = \Delta C_t$ (vaccinated) $- \Delta C_t$ (non-vaccinated) as described previously (Pridgeon et al., 2009). Data were analyzed by analysis of variance (ANOVA) using SigmaStat statistical analysis software (Systat Software, San Jose, CA).

3. Results

3.1. Evaluation of subtraction efficiency

The subtraction efficiency was evaluated by PCR analysis of the constitutively expressed gene 18S rRNA.

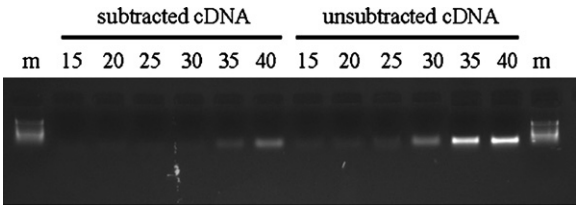

Fig. 1. Evaluation of subtraction efficiency. PCR analysis of the reduction of 18S rRNA abundance was performed using subtracted (lanes 1–6) and unsubtracted (lanes 7–12) cDNA samples as templates for 15 cycles (lanes 1 and 7), 20 cycles (lanes 2 and 8), 25 cycles (lanes 3 and 9), 30 cycles (lanes 4 and 10), 35 cycles (lanes 5 and 11), and 40 cycles (lanes 6 and 12). *m*: 100 bp DNA ladder.

As shown in Fig. 1, the amount of the 18S rRNA was significantly decreased after subtraction. Obvious bands were seen after 30 cycles in unsubtracted cDNAs but only after 35 and 40 cycles in subtracted cDNAs. The abundance of 18S rRNA was theoretically reduced by 2^5–2^{10} times, suggesting that cDNAs specific for vaccinated fish were enriched for about 2^5–2^{10} times by suppression hybridization.

3.2. Characteristics of the subtractive cDNA library

A total of 240 clones were obtained from the anterior kidney subtractive library. Of the 240 clones, 182 contained inserts. Sequencing results revealed that these 182 ESTs represented 57 different genes (Table 1). All ESTs listed in Table 1 have been deposited in the GenBank dbEST under accession numbers GO898766–GO898822. The most frequently detected clones were glycosyltransferase ($n = 8$), neutrophil cytosolic factor 2 ($n = 4$), Rab11A ($n = 4$), caldendrin ($n = 4$), and LAMP5 ($n = 4$). Out of the 57 ESTs identified from the subtractive library, 19 shared homology with deposited *Danio rerio* proteins, seven shared homology with deposited *Ictalurus punctatus* proteins, and 6 shared homology with deposited *Salmo salar* proteins (Table 1). The biggest insert size was 842 bp (GO898807) and the smallest insert size was 119 bp (GO898781). The average insert size of the 57 ESTs was 387 bp (Table 1).

3.3. Expression profiling of the 57 ESTs in catfish after E. ictaluri vaccination

To determine whether the expression levels of the 57 ESTs isolated from the subtractive library were upregulated in *E. ictaluri* vaccinated catfish, gene-specific primers for the 57 ESTs were designed (Table 2) for relative QPCR experiments. QPCR results revealed that 43 ESTs were induced at least 2-fold higher in all three vaccinated fish compared to that in unvaccinated control fish (Table 3). Of the 43 upregulated genes, five were consistently upregulated greater than 10-fold (Table 3), including two highly upregulated (>20-fold) genes: glycosyltransferase (GTFase) and Toll-like receptor 5 (TLR5) (Fig. 2A). The transcriptional levels of GTPase 1, coatomer protein complex zeta 1 (CPCZ1), and type II arginine deiminase (ADI2) were consistently induced greater than 10-fold (Table 3). MHC class I α chain and transposase were

Table 1
List of genes isolated from the *E. ictaluri* vaccinated *vs.* non-vaccinated catfish anterior kidney subtractive cDNA library.

No.	Accession no.	Protein homology	Protein accession no.	Organism	Identities (%)	Score bits	e value	Insert size (bp)
1	GO898766	Glycosyltransferase	CAL56729	*Ostreococcus taur*	56%	33.9	4.9	291
5	GO898767	DNA topoisomerase I	ZP_02330037	*Paenibacillus*	33%	33.5	6.2	432
6	GO898768	Ring finger 144B	NP_001107142	*Xenopus tropicalis*	88%	134	2e−30	317
7	GO898769	NADH dehydrogenase 1 alpha subcomplex 4	XP_001234601	*Gallus gallus*	89%	86	1e−13	338
20	GO898770	Propionyl Coenzyme A carboxylase β	CAQ13492	*Danio rerio*	89%	163	5e−39	243
26	GO898771	Neutrophil cytosolic factor 2	BAF73667	*Cyprinus carpio*	76%	126	5e−28	248
27	GO898772	Surface antigen BspA-like	XP_001315236	*Trichomona vaginalis*	27%	33.1	8.1	475
28	GO898773	Ras-related protein Rab 11A	NP_001134031	*Salmo salar*	95%	144	3e−33	235
31	GO898774	Kruppel-like protein 1	XP_001944423	*Acyrthosiphon pisum*	36%	33.1	8.2	208
32	GO898775	c-mos	AAL55336	*Bachia flavescens*	48%	33.1	8.1	290
33	GO898776	Metacaspase-like protein	XP_680261	*Plasmodium berghei*	41%	33.9	6.0	525
34	GO898777	Coatomer protein complex, zeta 1	NP_571583	*Danio rerio*	100%	204	2e−51	316
41	GO898778	Slc3a2 protein	AAI59203	*Danio rerio*	61%	102	1e−20	249
42	GO898779	No homology	–	–	–			296
58	GO898780	Cytochrome *b*	AAY79027	*Ictalurus punctatus*	97%	253	5e−66	417
70	GO898781	Complement C4a	BAB03284	*Cyprinus carpio*	51%	41.2	0.031	119
77	GO898782	Splicing factor 3a, subunit 3	CAK04939	*Danio rerio*	99%	224	2e−57	427
78	GO898783	Glucose phosphate isomerase a	AAH83507	*Danio rerio*	91%	367	7e−100	739
81	GO898784	Adenosine deaminase-related growth factor	XP_643866	*Dictyostelium discoideum*	51%	36.2	0.97	471
83	GO898785	Huntingtin interacting protein K	XP_001922634'	*Danio rerio*	95%	182	1e−44	553
85	GO898786	Tumor necrosis factor, alpha-induced protein 2	XP_697839	*Danio rerio*	57%	156	1e−36	688

Table 1 (*Continued*)

No.	Accession no.	Protein homology	Protein accession no.	Organism	Identities (%)	Score bits	e value	Insert size (bp)
86	GO898787	Lysosomal-associated transmembrane protein 5	XP_001923185	*Danio rerio*	56%	55.1	2e−06	221
92	GO898788	Alpha-tubulin isotype M-alpha-2	NP_001098596	*Danio rerio*	99%	235	1e−60	353
93	GO898789	NADH dehydrogenase subunit 3	YP_913699	*Hypentelium nigricans*	88%	89.4	1e−16	372
101	GO898790	Caldendrin	XP_001511181	*Ornithorhynchus anatinus*	89%	92.8	2e−17	669
107	GO898791	Beta thymosin-like	NP_001124169	*Danio rerio*	92%	78.6	2e−13	272
120	GO898792	Granulin 1	AAI53544	*Danio rerio*	63%	97.1	5e−19	300
121	GO898793	CD83 antigen precursor	ACI67585	*Salmo salar*	29%	44.7	0.008	760
122	GO898794	Leukocyte cell-derived chemotaxin 2	NP_001041520	*Danio rerio*	74%	114	2e−24	212
125	GO898795	TrkA domain-containing protein	YP_001528048	*Desulfococcus oleovorans*	30%	35.4	2.3	556
126	GO898796	Ribosomal protein L5a	AAK95128	*Ictalurus punctatus*	100%	244	1e−63	363
127	GO898797	Neurobeachin-like 1	XP_001070093	*Rattus norvegicus*	78%	89.0	1e−16	169
128	GO898798	Multisubunit cleavage/polyadenylation specificity factor subunit A	XP_730560	*Plasmodium yoelii*	26%	34.3	7.6	646
134	GO898799	lysozyme g	ACN09851	*Salmo salar*	73%	214	2e−54	510
136	GO898800	CD45 precursor	AAV65851	*Ictalurus punctatus*	95%	349	4e−95	488
137	GO898801	Very large inducible GTPase 1	XP_6840863	*Danio rerio*	43%	105	2e−21	352
139	GO898802	SET translocation B	NP_958876	*Danio rerio*	98%	341	3e−92	690
148	GO898803	Tubulin alpha 8 like	CAQ14258	*Danio rerio*	97%	213	6e−54	317
151	GO898804	Eukaryotic translation initiation factor 3, subunit E	NP_001135167	*Salmo salar*	98%	164	2e−39	241
159	GO898805	Rapunzel 2	NP_001138713	*Danio rerio*	68%	129	8e−29	405
165	GO898806	Pre-mRNA processing factor 8	XP_002197327	*Taeniopygia guttata*	98%	199	7e−50	280
169	GO898807	Prostate stem cell antigen precursor-like/UPAR	ABD85498	*Ictalurus punctatus*	84%	247	1e−63	842
174	GO898808	DNA or RNA helicase of superfamily II	ZP_01235811	*Vibrio angustum S14*	65%	33.1	8.3	373
177	GO898809	Tubulin, alpha, ubiquitous	ABY89801	*Callithrix jacchus*	99%	237	3e−61	353
178	GO898810	Predicted protein	XP_002288130	*Thalassiosira pseudonana*	35%	47.0	6e−04	376
179	GO898811	Protein-arginine deiminasae type II-like	ABE98234	*Oreochromis mossambicus*	77%	133	6e−30	286
184	GO898812	Uroporphyrinogen decarboxylase	AAI08076	*Danio rerio*	84%	233	3e−58	318
195	GO898813	Tropomyosin alpha-3 chain	NP_001136186	*Salmo salar*	92%	113	8e−24	532
205	GO898814	MHC class I alpha chain	AAD08649	*Ictalurus punctatus*	87%	120	4e−26	188
210	GO898815	Matrix metalloproteinase-9	ABO86718	*Ictalurus punctatus*	95%	85.9	1e−15	126
212	GO898816	Sulfotransferase family, cytosolic, 2B, member 1a	XP_541518	*Canis familiaris*	28%	34.3	3.8	370
217	GO898817	Beta-actin	ABY62772	*Squalius alburnoides*	100%	304	1e−81	452
218	GO898818	Toll-like receptor 5	ABF74618	*Ictalurus punctatus*	96%	186	8e−46	434
219	GO898819	Solute carrier family 25, member 3 isoform 3	XP_002189856	*Taeniopygia guttata*	100%	42.4	0.014	455
223	GO898820	Transposase	ABV31710	*Salmo salar*	90%	224	2e−57	366
224	GO898821	Glutamic pyruvate transaminase 2	NP_001092227	*Danio rerio*	94%	113	5e−24	165
239	GO898822	NAD-dependent deacetylase sirtuin-5	ACI33678	*Salmo salar*	68%	34.7	2.8	243

Table 2
Gene-specific primers used in QPCR.

Clone no.	Accession no.	Forward primer (5′–3′)	Reverse primer (5′–3′)
1	GO898766	GCTTCCGAGGTCAGATGAGA	AGTGTTTTCATGGCCTCTGC
5	GO898767	CAGGGTCGACTGTTAAAGCA	GTGCGGGAACGACTGTTTAT
6	GO898768	TTCTTCTTGTGCCAGGGTTT	GGGGACATTTTCCTGAGACA
7	GO898769	CGGCCAGACTTCTAAATCCA	TGCGGTTTAGCCTGTTTTCT
20	GO898770	TCACCATCATCACCAGGAAG	TGATTCTCCTTTCCCCTGAA
26	GO898771	GGGTTTAACCGGAGCATCTT	ATTCGACCTGAGCTGCTGTT
27	GO898772	TTGTGGAATGCCTCTGACTG	TTTTGCCGTAGACCGAAGAC
28	GO898773	GCCATGACGGGTAGAGAAGA	CAGATCTGGGCTTTGATCGT
31	GO898774	ATGCCTTTTCTCACCACCTC	AACTCGTGCAAGGCTGAAAT
32	GO898775	GTTCATGCAGCATTCACACC	CCACAACACATCAGGTTCCA
33	GO898776	TGGAAAATGCAAACGAATCA	AAAGAAGGCAAGCAGAATGTG
34	GO898777	AGAAAAGGCCCTCCATGTTT	TTGCTCGAAGGCCTTACTGT
41	GO898778	ATCCTTTGCTTGGCATTGAG	TTTTTCAGCCAGTAGGCACA
42	GO898779	TTAATGAGTCGGTGCTGCTG	AATCCTGCCCTCCTTCAGTT
58	GO898780	GGAGGTTGGGAGAGAAAAGG	TCGCAGCAACACTACTCCAC
70	GO898781	CGGGAAGTGCTACAGCG	CCTAAAGCGCGTCTCAG
77	GO898782	ACGTCACCCAGATCGAAGAC	AAGGGGTTCTCAAGGTTGCT
78	GO898783	CTCCAGAGCATCACCACTCA	CTGCTCAATCTCAGCATCCA
81	GO898784	TGCAGATGCCTGCTTAAATG	AGCTGCTGGCTTTTATTGGA
83	GO898785	TCGAGTTCGGACTTGGAAAC	TTGGTCAGTGCAATCAAAGC
85	GO898786	TCATGTATGACCCAGCCTCA	CTTGATGGGGTGCATAGACA
86	GO898787	TCTGCGTGCTTGTTTTATGC	CAGGTGTCCATTCGCTGTTA
92	GO898788	TCAGATCGTGTCCTCCATCA	CTCAAAGCAGGCATTTGTGA
93	GO898789	AGAAGGGTGTAAGCGGGAGT	AAAAGCTCTCCCCCTACGAA
101	GO898790	CCAGCCAGCAGAAAGTAAGG	AGGGAAGCCATGAGGAAACT
107	GO898791	GACAAACCCAATCTCG	CCTGCTTCTCCTGTTCG
120	GO898792	ACATGCTGCAGGTCTCCTTT	TCTGAATGGTGTTGCTCTGC
121	GO898793	AACCGACAAGAGACCACAGAA	CAGCCCTTTGACTCTGGAAT
122	GO898794	AAACAACGCCATCAACAATG	GTTCGACTTGTCGCACATCT
125	GO898795	GTGACAGCAGGGGTTTTTGT	CGGCTTTAGGGTGGATATGA
126	GO898796	AGTGCCTTCACGTGCTACCT	GTTCAGGCCCAGAATGTGTT
127	GO898797	CACCAAGGAGAGCAGTCACA	TCTCCTGCAGACACCTGAGA
128	GO898798	AAACACGTGCAGGAGGAACT	CGTGCTTCGTTAGCCTTTTC
134	GO898799	GAGCCTGGAACAGTGAGGAG	GTGGTGCCTCTGTCCATTTT
136	GO898800	GTTCGGCAGTTGCAGATTTT	TGCACGTTACTTTCCAGGTG
137	GO898801	TCCATGAGCACAGTGGAGAG	AAGGCTCATCTTGGGGTTTT
139	GO898802	CTCTGCTTGGTGAGGAGGAC	AGTCCTTTCCAGCCTTCCAT
148	GO898803	TGATGGCAATATGCCAAGTG	CTGCATCTTCCTTCCCAGAG
151	GO898804	CTGAGCGCTGTCAAAGTCAA	ACCTAACCACAGCGGTCATC
159	GO898805	CACTTCTCCCTGACCTCGTC	AGTCTTTTTGGGAGGCCACT
165	GO898806	TCCTCCAGATAACCCACAGG	ATGATGGTCTTCTCGCCATC
169	GO898807	TGCATCAGTGCATCAGTTCA	GGACGATCATGAGGAGGAAA
174	GO898808	AAACGCTACGGAGCTTTGAA	CACCTGGTTCCATAGAGGACA
177	GO898809	TCAGATCGTGTCCTCCATCA	CTCAAAGCAGGCATTTGTGA
178	GO898810	TCAGATCGTGTCCTCCATCA	CTCAAAGCAGGCATTTGTGA
179	GO898811	TGAGACGTGCTCTTTGCTTG	TCTCACAGCTCAAGGTTCCA
184	GO898812	CAAGGGTCAATGGTCCAATC	GACATCGCACGCAGAGTAAA
195	GO898813	CGCCTTATCATATGCGGTCT	CCCCACCTTCTACCCACTTT
205	GO898814	CACCAGGAATAAATTTCCCAGA	CTGTGTCTGCGTGTTCCAGT
210	GO898815	ACATCTTATTAGTAAGAGGGTCC	ACTACTGGAAGTCCTCAAATCG
212	GO898816	CAACATGAGAGCGAAGTGGA	AGCAGATGGACAGCACCTCT
217	GO898817	CTCGAAGTCAAGGGCAACAT	CGTGATGGACTCTGGTGATG
218	GO898818	TTGGAAGCGCTACAAATCCT	ACCCGGAGGTTGAATAATCC
219	GO898819	CAGAAACAGCTTTGCACCTG	CTCATTCCCCTGTCCATCAC
223	GO898820	ACCGTCAAATCTTGGGTGAG	GCAACTCGAACCTTCAGCTC
224	GO898821	AAACAGCACTGCCAGC	ATACACCTCATCGGCCA
239	GO898822	CCAAAAAGGAGGCACACTTG	GCCAAAGATGCCAGAATACC

upregulated greater than 10-fold in two of the three vaccinated fish. The transcription level of NAD-dependent deacetylase sirtuin-5 was induced greater than 8-fold in two of the three vaccinated fish. The transcriptional levels of c-mos, adenosine deaminase-related growth factor, huntingtin interacting protein K, and tumor necrosis factor α-induced protein 2 were consistently upregulated greater than 5-fold (Fig. 2B). Other genes that were consistently induced greater than 3-fold and included the following: Kruppel-like protein 1, matrix metalloproteinase-9, DNA topoisomerase I, propionyl coenzyme A carboxylase β, netrophil cytosolic factor 2, surface antigen BspA-like, complement C4a, granulin 1, glutamic pyruvate transaminase 2, rapunzel 2, prostate stem cell antigen precursor-like/plasminogen activator urokinase receptor, DNA or RNA helicase of superfamily II, a predicted protein with

Table 3
Modified live *E. ictaluri* vaccine induced expression of the 57 genes.

Gene/clone	Accession no.	Putative protein	Vaccine induced expression ($2^{\Delta\Delta C_t}$) (fold)		
			$2^{\Delta\Delta C_t-1}$	$2^{\Delta\Delta C_t-2}$	$2^{\Delta\Delta C_t-3}$
1	GO898766	DNA topoisomerase I	72.05	101.48	32.11
5	GO898767	Ring finger 144B	5.35	5.68	3.45
6	GO898768	NADH dehydrogenase 1 alpha subcomplex 4	2.46	2.27	2.98
7	GO898769	Propionyl coenzyme A carboxylase β	1.79	2.07	3.16
20	GO898770	Neutrophil cytosolic factor 2	3.84	3.31	3.61
26	GO898771	Surface antigen BspA-like	3.53	5.15	4.77
27	GO898772	Ras-related protein Rab 11A	3.07	3.38	4.79
28	GO898773	Kruppel-like protein 1	1.85	2.63	5.22
31	GO898774	c-mos	4.25	4.6	9.06
32	GO898775	Metacaspase-like protein	6.71	6.45	7.39
33	GO898776	Coatomer protein complex, zeta 1	2.94	2.27	3.24
34	GO898777	Slc3a2 protein	10.93	18.25	18.25
41	GO898778	No homology	2.67	3.49	5.56
42	GO898779	Cytochrome *b*	2.53	2.91	3.96
58	GO898780	Complement C4a	3.14	3.04	2.92
70	GO898781	Splicing factor 3a, subunit 3	3.79	4.94	6.77
77	GO898782	Glucose phosphate isomerase a	2.12	2.6	2.34
78	GO898783	Adenosine deaminase-related growth factor	1.72	2.14	3.07
81	GO898784	Huntingtin interacting protein K	5.35	6.13	6.8
83	GO898785	Tumor necrosis factor, alpha-induced protein 2	5.03	5.58	8.11
85	GO898786	Lysosomal-associated transmembrane protein 5	7.84	6.06	11.16
86	GO898787	Alpha-tubulin isotype M-alpha-2	2.89	3.73	4.91
92	GO898788	NADH dehydrogenase subunit 3	1.74	2.11	3.75
93	GO898789	Caldendrin	5.28	4.13	2.82
101	GO898790	Beta thymosin-like	1.83	3.14	3.31
107	GO898791	Granulin 1	0.91	1.17	1.56
120	GO898792	CD83 antigen precursor	3.21	4.36	5.12
121	GO898793	Leukocyte cell-derived chemotaxin 2	2.61	3.47	8.25
122	GO898794	TrkA domain-containing protein	3.68	3.12	6.08
125	GO898795	Ribosomal protein L5a	1.42	2.2	2.48
126	GO898796	Neurobeachin-like 1	1.02	1.09	1.46
127	GO898797	Multisubunit cleavage/polyadenylation specificity factor subunit A	2.91	4.61	4.55
128	GO898798	Lysozyme g	2.79	4.87	6.45
134	GO898799	CD45 precursor	2.53	2.17	3.17
136	GO898800	Very large inducible GTPase 1	1.55	9.03	3.59
137	GO898801	SET translocation B	15.81	30.8	25.11
139	GO898802	Tubulin alpha 8 like	1.51	4.08	3.4
148	GO898803	Eukaryotic translation initiation factor 3, subunit E	2.77	8.85	8.97
151	GO898804	Rapunzel 2	2.22	5.84	3.58
159	GO898805	Pre-mRNA processing factor 8	3.87	9.32	5.92
165	GO898806	Prostate stem cell antigen precursor-like/UPAR	2.5	5.17	4.86
169	GO898807	DNA or RNA helicase of superfamily II	3.32	6.41	4.89
174	GO898808	Tubulin, alpha, ubiquitous	3.66	6.04	6.34
177	GO898809	Predicted protein	2.18	3.22	4.52
178	GO898810	Protein-arginine deiminasae type II-like	3.12	3.39	3.95
179	GO898811	Uroporphyrinogen decarboxylase	11.15	17.88	11.43
184	GO898812	Tropomyosin alpha-3 chain	0.54	0.46	1.38
195	GO898813	MHC class I alpha chain	3.16	6.52	2.7
205	GO898814	Matrix metalloproteinase-9	17.53	26.54	4.71
210	GO898815	Sulfotransferase family, cytosolic, 2B, member 1a	4.2	4.71	4.21
212	GO898816	Beta-actin	1.14	1.11	1.58
217	GO898817	Toll-like receptor 5	1.12	1.16	1.52
218	GO898818	Solute carrier family 25, member 3 isoform 3	31.61	43.26	21.63
219	GO898819	Transposase	1.35	2.24	2.23
223	GO898820	Glutamic pyruvate transaminase 2	20.25	7.73	10.48
224	GO898821	NAD-dependent deacetylase sirtuin-5	5	6.77	3.59
239	GO898822		19.46	8.52	3.64

unknown function, and leucocyte derived chemotaxin 2 (LECT2). Seventeen genes slightly upregulated 2–3-fold included the following: ringer finger 144B, metacaspase-like protein, slc3a2 protein, cytochrome *b*, splicing factor 3a3, lysosomal-associated transmembrane protein 5 (LAMP5), NADH dehydrogenase subunit 3, CD83 antigen precursor, neurobeachin-like 1 multisubunit cleavage/polyadenylation specificity factor subunit A, lysozyme g, tubulin alpha 8, eukaryotic translation initiation factor 3E, pre-mRNA processing factor 8, tubulin alpha ubiquitous, tropomyosin alpha-3 chain, and one novel protein (Fig. 2C). Nine genes induced greater than 2-fold in two of the three

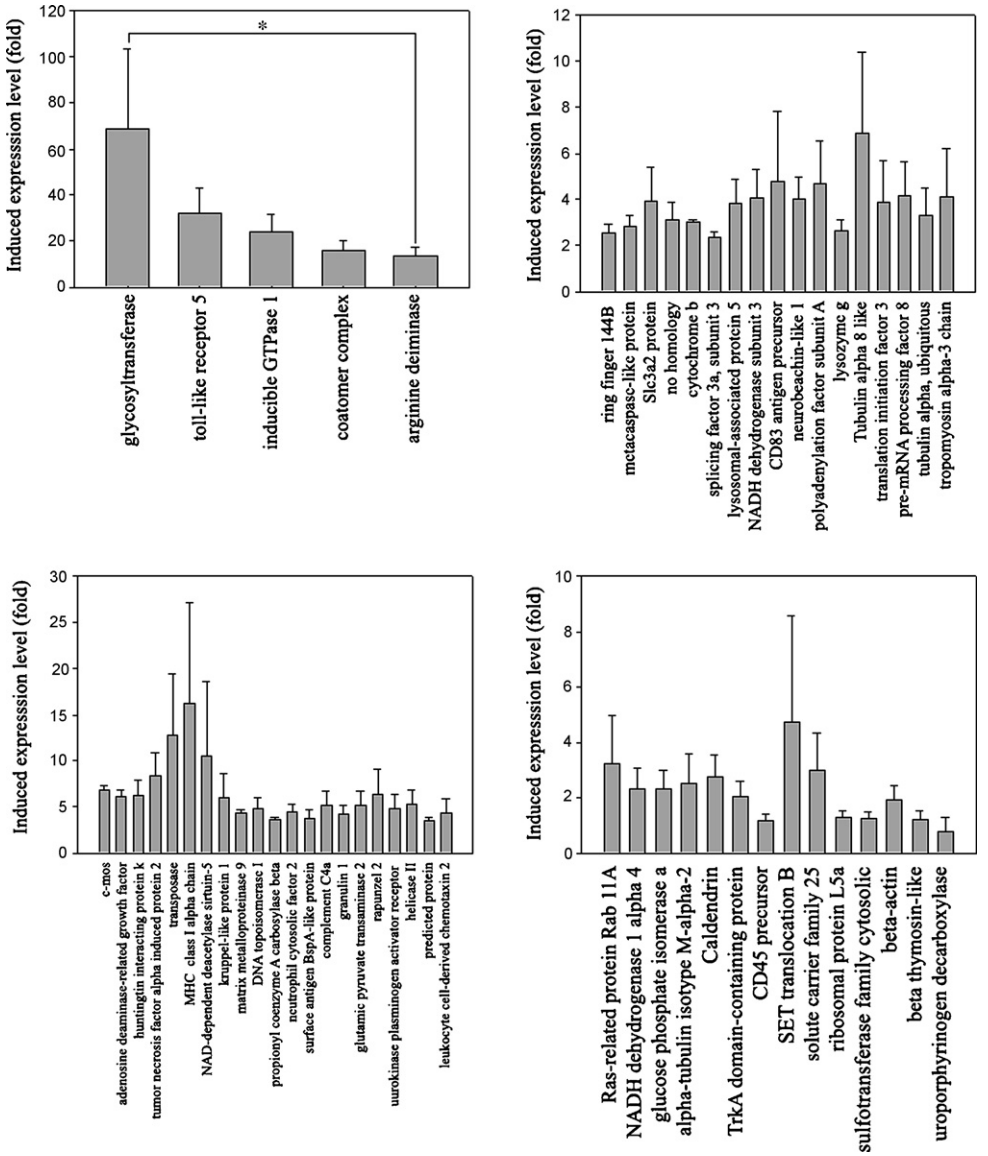

Fig. 2. Transcriptional regulation of the 57 genes isolated from the channel catfish head kidney subtractive library. (A) Transcription levels of 5 highly upregulated (>10-fold) genes induced by *E. ictaluri* vaccination; (B) transcription levels of 21 moderately upregulated ($3 < x < 10$-fold) genes induced by *E. ictaluri* vaccination; (C) transcription levels of 17 slightly upregulated ($2 < x < 3$-fold) genes induced by *E. ictaluri* vaccination; (D) transcription levels of 14 genes not significantly induced by *E. ictaluri* vaccination (<2-fold). The relative transcriptional levels of different genes were determined by subtracting the cycle threshold (C_t) of the sample by that of the 18S rRNA, the calibrator or internal control, as per the formula: $\Delta C_t = C_t$ (sample) $- C_t$ (calibrator). The relative expression level of the specific gene in *E. ictaluri* RE-33 vaccinated fish compared to that in non-vaccinated fish was then calculated by the formula $2^{\Delta\Delta C_t}$ where $\Delta\Delta C_t = \Delta C_t$ (vaccinated) $- \Delta C_t$ (non-vaccinated). Data are presented as means ± SEM. Differences were considered statistically significant when $p < 0.05$ and represented by an asterisk.

vaccinated fish included the following: Ras-related protein Rab-11A, NADH dehydrogenase 1 alpha subcomplex 4, glucose phosphate isomerase a, alpha-tubulin isotype M-alpha-2, caldendrin, TrkA domain-containing protein, CD45 precursor, SET translocation (myeloid leukemia-associated) B, and solute carrier family 25, member 3 isoform 3. Five genes that were induced less than 2-fold in all three vaccinated fish were the following: ribosomal protein L5a, sulfotransferase family cytosolic 2B member 1a, beta-actin, beta thymosin-like, and uroporphyrinogen decarboxylase (Fig. 2D).

3.4. Classification of the E. ictaluri vaccination-upregulated genes

The 57 genes isolated from the subtractive library were classified in terms of their putative functions (Table 4). Half of the genes identified were either involved in immune-response or metabolism (Fig. 3). The major portion (28%) of the 57 genes identified were immune-related genes, including MHC class I alpha chain, surface antigen BspA-like protein/NK-lysin type 3, complement C4a, leukocyte cell-derived chemotaxin 2 (LECT2), CD83 antigen pre-

Table 4
Putative function of *E. ictaluri* vaccination-upregulated genes.

Category	Protein	Putative function related to infection
Immune-related protein (16)	Glycosyltransferase	Immune response-T cell apoptosis
	Similar to very large inducible GTPase 1	Mediate in innate and adaptive immunity
	MHC class I alpha chain	Antiviral immunity
	Surface antigen BspA-like/NK-lysin type 3	Cell attachment and invasion
	Complement C4-1	Acute infection
	Granulin 1	Phagocytosis
	Prostate stem cell antigen precursor-like/urokinase plasminogen activator receptor (uPAR or PLAUR)	Modulate the development of protective immunity
	Leukocyte-derived chemotaxin 2 (LECT2)	Infection induced gene
	CD83 antigen precursor	Dendritic cell maturization
	Lysozyme g	Bactericidal activity
	c-mos	Phagocytosis
	Matrix metalloproteinase-9	Host defense
	Neutrophil cytosolic factor 2	Phagocytosis
	Toll-like receptor 5	Innate immunity
	CD45 precursor	Type I inteferon production
	Similar to tumor necrosis factor, alpha-induced protein 2	Innate immunity
Metabolism-related protein (12)	NAD-dependent deacetylase sirtuin-5	Cellular metabolism
	Similar to sulfotransferase family, cytosolic, 2B, member 1 isoform a	Mitochondria burst/Metabolism
	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4,	Phagocytosis/oxidative stress response
	Cytochrome *b*	Mitochondria burst/Bacteria pathogenesis
	Splicing factor 3a, subunit 3	Xenobiotics detoxification
	Propionyl Coenzyme A carboxylase, beta polypeptide	Metabolism
	Glutamic pyruvate transaminase (alanine aminotransferase) 2	metabolism
	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3 isoform 3	metabolism
	Uroporphyrinogen decarboxylase	metabolism
	NADH dehydrogenase subunit 3	metabolism
	Glucose phosphate isomerase a	metabolism
	Protein-arginine deiminasae type II-like	metabolism
Apoptosis-related protein (6)	Slc3a2 protein	Apoptosis-related/CD98HC; Secretory pathways
	Beta thymosin-like	Apoptosis
	similar to huntingtin interacting protein K	Apoptosis
	Metacaspase-like protein	Apoptosis
	Similar to Kruppel-like protein 1	Apoptosis
	Ring finger 144B	Apoptosis
Cell structure, growth and maintenance (8)	Beta-actin	Cell structure
	Similar to alpha-tubulin isotype M-alpha-2	Cell structure
	Tubulin, alpha 8 like	Cell structure
	Tubulin, alpha, ubiquitous (predicted)	Cell structure
	Tropomyosin alpha-3 chain	Actin-related
	SET translocation (myeloid leukemia-associated) B	Nucleosome assembly
	ribosomal protein L5a	
	adenosine deaminase-related growth factor	Cell proliferation
Endocytosis, vesicular, and lysosome trafficking (3)	Similar to lysosomal-associated transmembrane protein 5 (lysosomal-associated multitransmembrane protein 5) (Retinoic acid-inducible E3 protein)	Lysosome trafficking
	coatomer protein complex, zeta 1	Vesicle trafficking
	Ras-related protein Rab-11A	Endocytosis and trafficking
Transcription and translation related protein (3)	PRP8 pre-mRNA processing factor 8 homolog	Transcription regulation
	Eukaryotic translation initiation factor 3, subunit E, a	Translation
	Multisubunit cleavage/polyadenylation specificity factor subunit A	Transcription
Bacterial proteins (3)		
	DNA topoisomerase I	bacteria
	DNA or RNA helicase of superfamily II	bacteria
	TrkA domain-containing protein	Bacteria

Table 4 (*Continued*)

Category	Protein	Putative function related to infection
Signal transduction (1)	Caldendrin	Calcium signaling
Functionally unknown proteins (5)	No homology	unknown
	Unnamed protein product	unknown
	Transposase	unknown
	Rapunzel 2	unknown
	Predicted protein	unknown

cursor, lysozyme g, CD45 precursor, and Toll-like receptor 5, glucose phosphate isomerase a, and TNF alpha-induced protein 2 (TNFAIP2). Another major group (21%) of the identified genes was related to metabolism, NADH dehydrogenase subunit 3 and cytochrome *b*. Six genes (10%) were related to apoptosis, including huntingtin interacting protein K and ring finger 144B. Eight genes (14%) were related to cell growth and maintenance, including beta-actin and alpha-tubulin. Three genes (5%) were related to endocytosis and lysosome trafficking, including lysosomal-associated transmembrane protein 5 (LAMP5) and Ras-related protein Rab-11A. Three genes (5%) were related to gene regulation, including pre-mRNA processing factor 8 and eukaryotic translation initiation factor 3. One gene (2%), caldendrin, was involved in calcium signaling. The functions of five genes (9%) are currently unknown.

4. Discussion

Enteric septicemia of catfish (ESC) is the most prevalent disease affecting farm-raised channel catfish (Wagner et al., 2002) and live attenuated *E. ictaluri* vaccines have been shown to be efficacious in protecting catfish through immersion (Wise et al., 2000; Shoemaker et al., 1999, 2002, 2007). However, it is not clear whether vaccination will induce consistent molecular responses in channel catfish after exposure to *E. ictaluri* vaccine. Therefore, the purpose of this study was to isolate upregulated genes in channel catfish after modified live *E. ictaluri* vaccination without any preconception of their identities. We sampled fish at 48 h post-vaccination because kinetic studies have revealed that pathogen loads are peaked on day 2 in channel catfish and that Toll-like receptor 3 is significantly induced on day 2 in channel catfish after immersion challenge with *E. ictaluri* (Bilodeau & Waldbieser, 2005). Using subtractive cDNA hybridization and QPCR, Toll-like receptor 5 (TLR5) was identified to be consistently upregulated greater than 20-fold in all three vaccinated catfish. Toll-like receptor 5 is a well-characterized receptor in both mammals and fish that recognizes flagellin of bacteria (Gewirtz et al., 2001; Tsujita et al., 2004). The expression of channel catfish TLR5 in response to virulent strain of *E. ictaluri* has been demonstrated to be significantly upregulated in the anterior kidney and in the liver following ESC infection (Bilodeau and Waldbieser, 2005; Bilodeau et al., 2006; Peatman et al., 2008). The identification of TLR5 from the subtractive library without any preconception of TLR5's identity confirms that suppression subtractive hybridization is a powerful technique to identify upregulated genes in one mRNA population *vs.* another.

EST GO898766 was consistently upregulated greater than 20-fold in all three vaccinated fish. At protein level, GO898766 shared 56% identity with glycosyltransferase of *Ostreococcus taur*, a free-living eukaryote. However, the e value of 4.9 in the blast search suggested that GO898766 might be a functionally unknown protein. The function of glycosyltransferase is to catalyze the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, forming glycosidic bonds. The acceptor molecule can be a lipid, a protein, a heterocyclic compound, or another carbohydrate residue. Recently, a putative glucosyltransferase designated biofilm-associated

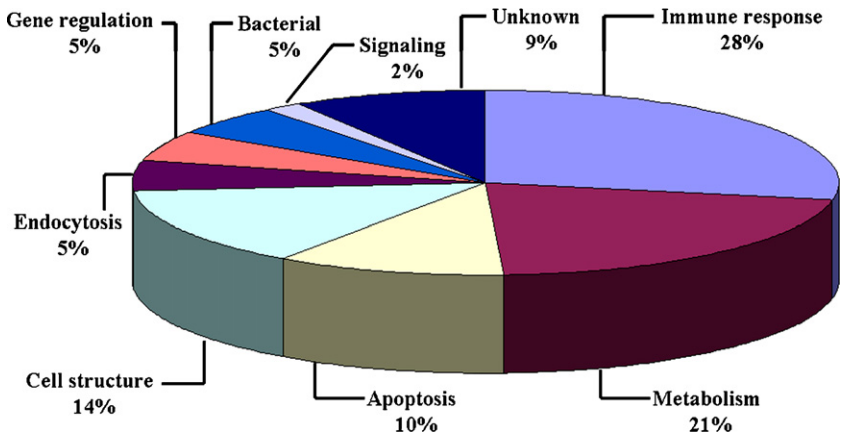

Fig. 3. Classification of the differentially expressed genes identified from the subtractive library. Pie charts representing the distribution of the 57 identified genes according to their putative biological function.

glycolipid synthesis A (*bgsA*) in bacterium *Enterococcus faecalis* has been demonstrated to synthesize DGlcDAG, a glycolipid and lipoteichoic acid precursor involved in biofilm accumulation, adherence to host cells, and virulence *in vivo* (Theilacker et al., 2009), suggesting that this EST might be a virulence gene in *E. ictaluri*. It is also possible that GO898766 is a host immune-related gene since it has been suggested that the expression of glycosyltransferase can modulate cell death during T cell development and function through controlling susceptibility to galectin-1 (Galvan et al., 2000). The function of galectin-1 is to induce apoptosis of immature thymocytes and activate T cells (Galvan et al., 2000). 5′- and 3′-rapid amplification of cDNA ends will be merited to understand the true identity of ESTGO898766.

EST GO898801 was upregulated greater than 15-fold in all three vaccinated fish. GO898801 shared 43% identity with a very large inducible GTPase 1 of zebra fish *Danio rerio* at protein level (e value = 2e−21). Very large inducible GTPase 1 has been reported to be inducible by interferons, important players in both innate immunity and adaptive immunity (Klamp et al., 2003). Recently, it has been demonstrated that the expression of guanylate-binding protein-1 (GBP-1), an interferon inducible large GTPase, is upregulated in colonic epithelia of individuals with inflammatory bowel disease (Schnoor et al., 2009), suggesting that this very large inducible GTPase 1 might be a specific response to *E. ictaluri* vaccination and/or infection.

EST GO898777 was upregulated greater than 10-fold in all three vaccinated fish. GO898777 shared 100% identity with coatomer protein complex zeta 1 of zebra fish *Danio rerio* at protein level (e value = 2e−51). Coatomer protein complex is involved in endocytosis and vesicle trafficking (Maier et al., 2001). It has been demonstrated that coatomer protein complex is necessary for the maintenance and/or localization of a critical pool of elements of the phagocytic machinery (Hackam et al., 2001), suggesting that the upregulation of coatomer protein complex zeta 1 might play an important role in phagocytosis of *E. ictaluri* in the channel catfish to survive ESC.

EST GO898811 was upregulated greater than 11-fold in all three vaccinated fish. GO898811 shared 77% identity with type II arginine deiminase of Mozambique tilapia *Oreochromis mossambicus* at protein level (e value = 6e−30). The function of arginine deiminase (ADI) is to catalyze the irreversible hydrolysis of arginine to citrulline and ammonia (Shirai et al., 2001). ADI has been shown to be able to inhibit lipopolysaccharide (LPS)-induced upregulation of inducible nitric oxide synthase and the production of nitric oxide in murine macrophages in dose-dependent manner (Kim et al., 2007), suggesting that ADI upregulation in the channel catfish might be a specific response to *E. ictaluri* infection and/or vaccination.

EST GO898814 was upregulated greater than 17-fold in two of the three vaccinated fish. GO898814 shared 87% identity with major histocompatibility complex (MHC) class I alpha chain of channel catfish *Ictalurus punctatus* at protein level (e value = 4e−26). MHC plays an important role in the immune system. Proteins encoded by the MHC are expressed on the surface of cells, displaying both self antigens (peptide fragments from the cell itself) and nonself antigens (e.g. fragments of invading microorganisms) to T cell that has the capacity to kill or co-ordinate the killing of pathogens and infected or malfunctioning cells. Upregulation of MHC class I alpha chain has been reported in the liver of blue catfish 3 days post-challenge by *E. ictaluri* (Peatman et al., 2008), suggesting that the upregulation of MHC class I alpha chain might play an important role in the host immune response to *E. ictaluri* infection and/or vaccination.

EST GO898822 was upregulated greater than 8-fold in two of the three vaccinated fish. GO898822 shared 68% identity with NAD-dependant deacetylase sirtuin-5 protein of salmon *Salmo salar*. However, the e-value was only 2.8, suggesting that sirtuin-5 sequence in channel catfish might be very different from that in salmon. The enzymes of the sirtuin family of nicotinamide-adenine-dinucleotide-dependent protein deacetylases are emerging key players in nuclear and cytosolic signaling, but also in mitochondrial regulation and aging. Sirtuin-5 has been demonstrated to be able to deacetylates carbamoyl phosphate synthetase 1 and regulates the urea cycle in mice (Nakagawa et al., 2009). Sirtuin 5 has also been demonstrated to be able to deacetylate cytochrome *c*, a protein of the mitochondrial intermembrane space with a central function in oxidative metabolism as well as apoptosis initiation (Schlicker et al., 2008). The upregulation of sirtuin 5 in channel catfish in response to *E. ictaluri* vaccination suggests that sirtuin 5 might also play an important role in host immune defense.

EST GO898772 was upregulated greater than 3-fold in all three vaccinated fish. GO898772 shared 27% identity with surface antigen BspA-like protein of eukaryotic pathogen *Trichomona vaginalis* at protein level (e value = 8.1). It has been reported that epithelial cell attachment and invasion by bacteria *Tannerella forsythia* are dependent on the BspA protein (Inagaki et al., 2006). However, at nucleotide level, GO898772 shared 76% identity with NK-lysin type 3 gene of the channel catfish (e value = 5e−38). NK-lysin is a 9-kDa polypeptide that was originally isolated from porcine intestinal tissue based on its antibacterial activity. NK lysine has been reported to be able to bind LPS from *Escherichia coli*, *Pseudomonas aeruginosa*, and different strains of *Salmonella enterica* and lyse lymphoma cells (Andersson et al., 1999), suggesting that NK-lysin type 3 may be a natural LPS-binding protein that may participate in the endogenous defense response in the channel catfish in response to *E. ictaluri* vaccination.

EST GO898781 was upregulated greater than 3-fold in all three vaccinated fish. GO898781 shared 51% identity with complement C4a of common carp *Cyprinus* carpio (e value = 0.031). The complement system is an important humoral defense mechanism that plays a vital role against microbial agents, inflammatory response control, and immunocomplex clearance. Classical complement pathway activation is antibody-dependent. The C4 component participates in the initial step of activation, and C4 expression is determined by 2 pairs of allotypes: C4a and C4b (Samano et al., 2004; Rambach et al., 2008). It has been reported that C4a levels was significantly higher in

acute Lyme disease patients than in tick bite and healthy control groups (Shoemaker et al., 2008). In *E. ictaluri* challenged blue catfish liver, C4b expression has been reported to be upregulated for 3.9-fold compared to non-challenged control (Peatman et al., 2008), suggesting that the upregulation of C4a in channel catfish might serve as a molecular marker for *E. ictaluri* infection and/or vaccination.

EST GO898792 was upregulated greater than 3-fold in all three vaccinated fish. GO898792 shared 63% identity with granulin-1 protein of zebra fish *Danio rerio* (e value = 5e−19). The granulins are small proteins of about 6 kDa that are derived from a larger precursor of approximately 590 amino acids (Bhandari et al., 1992, Plowman et al., 1992). Both the 6-kDa peptide and the intact precursor have been found to modulate cell growth (Bateman and Bennett, 1998). It has been reported that equine granulin E is able to kill *Streptococcus zooepidemicus* and sustain a greater than 99.8% decrease in CFU per milliliter after a 2-h exposure to 100 μg/mL (approximately 15 μM) of granulin E (Couto et al., 1992), suggesting that the upregulation of granulin-1 in vaccinated channel catfish might be involved in host immune defense against *E. ictaluri* vaccination and/or infection.

EST GO898807 was upregulated 3–6-fold in all three vaccinated fish. GO898807 shared 84% identities with prostate stem cell antigen precursor-like protein of channel catfish (e value = 1e−63). GO898807 also shared 39% identity with urokinase plasminogen activator receptor of zebra fish with an e value of 2e−08. The urokinase plasminogen activator receptor (uPAR) is expressed at the cell surface of inflammatory cells and plays an important role in neutrophil migration. It has been reported that uPAR is crucially involved in host defense through phagocytosis during *E. coli* induced acute pyelonephritis in mice (Roelofs et al., 2006), suggesting that upregulation of uPAR might play an important role in hose defense against *E. ictaluri* vaccination and/or infection.

EST GO898794 was upregulated 3–6-fold in all three vaccinated fish. GO898794 shared 74% identity with leukocyte cell-derived chemotaxin 2 (LECT2) of zebra fish (e value = 2e−24). LECT2 was initially isolated as a possible chemotatic factor for neutrophils (Yamagoe et al., 1996) and subsequently demonstrated to be involved in liver regeneration (Saito et al., 2004), carcinogenesis (Ovejero et al., 2004) and NK Tcell homeostasis (Saito et al., 2004). Recently, it has been reported that LECT2 is induced 51-fold by *Staphylococcus aureus* and 1344-fold by *Aeromonas salmonicida* infection in zebra fish (Lin et al., 2007), suggesting that upregulation of LECT2 might play an important role in anti-infection and anti-inflammation.

EST GO898793 was upregulated 2.6–8-fold in three vaccinated fish. GO898793 shared 29% identity with CD83 antigen precursor of atlantic salmon *Salmo salar* (e value = 0.008). CD83, an evolutionarily well-conserved highly glycosylated type 1 transmembrane glycoprotein, is a highly specific marker for activated dendritic cells (DCs) in human. The function of CD83 is to regulate lymphocyte maturation, activation and homeostasis (Breloer and Fleischer, 2008). It has been reported that CD83 is rapidly upregulated on murine B cells, reaching maximal expression 6 h after either Toll-like receptor (TLR) engagement by lipopolysaccharide (LPS) or B cell receptor (BCR) ligation (Kretschmer et al., 2007). It has been reported that monocyte-derived dendritic cells is able to recognize and respond to the essential viral glycoproteins, leading to the upregulation of CD40, CD83, and CD86 in response to herpes simplex virus-1 infection (Reske et al., 2008), suggesting that upregulation of CD83 might play important functional role in the channel catfish in response to *E. ictaluri* infection and/or vaccination.

EST GO898799 was upregulated 2–3-fold in three vaccinated fish. BLAST homology search revealed that GO898799 shared 73% identity with lysozyme g of atlantic salmon *Salmo salar* (e value = 2e−54). Lysozyme functions as a crucial biodefense effector against the infection of bacterial pathogens in innate immunity. It has been reported that the expression of g-type lysozyme is significantly upregulated in the vaccinated Atlantic cod, Gadus morhua after intraperitoneal vaccination of heat-killed *Listonella anguillarum* (Caipang et al., 2008), suggesting that lysozyme g upregulation might be a specific immune response to *E. ictaluri* vaccination.

Other genes that were upregulated greater than 3-fold in all three vaccinated fish include ESTs that shared identities with c-mos, adenosine deaminase-related growth factor, kruppel-like protein, matrix metalloproteinase-9, and neutrophil cytosolic factor 2. The proto-oncogene c-mos regulates macrophage differentiation in a dose-dependent manner (Kurata et al., 1989). Adenosine deaminase-related growth factor plays an important role in cell proliferation (Zurovec et al., 2002). High steady state expression of kruppel-like protein has been reported in Ag-specific $CD8^+$ memory T cells, critical for controlling viral as well intracellular and parasitic infections (Grayson et al., 2001). Matrix metalloproteinase-9 (MMP-9) is involved in the migration of inflammatory cells across the extracellular matrix, as well as tissue remodeling (Delclaux et al., 1996; Opdenakker et al., 2001). MMP-9 was first identified in neutrophils, but can also be expressed by various other cell types such as monocytes/macrophages, lymphocytes, and endothelial cells. MMP-9 is not produced constitutively, but needs a trigger to be expressed (Opdenakker et al., 2001). However, Yeh and Klesius (2008) have suggested that MMP-9 is constitutively expressed in restricted tissues including the head kidney of channel catfish. It has been demonstrated that LPS, the major constituent of the outer cell wall of Gram-negative bacteria and the principal mediator of inflammatory responses to these pathogens, is able to induce the release of MMP-9 by neutrophils and monocytes in vitro (Masure et al., 1991; Opdenakker et al., 1991). Moreover, in mice, *E. coli* LPS administration has led to a quick release of MMP-9 into the circulation, with peak values as soon as 1 h after injection (Dubois et al., 2002), suggesting that the upregulation of MMP-9 in the channel catfish might be specifically induced by *E. ictaluri* vaccination. Neutrophil cytosolic factor 2 (NCF2) is a protein encodes $p67^{phox}$, a subunit of the multiprotein enzyme complex NADPH oxidase. NCF2 has been shown to be responsible for the

generation of superoxides. The phagocyte NADPH oxidase has a crucial role in innate immunity by specifically reducing molecular oxygen to superoxide. Superoxide anions give rise to numerous toxic reactive oxygen species that are used as microbicidal agents against pathogens, contributing to the respiratory burst typically seen in phagocytic cells. Upon activation by opsonized microbes or inflammatory mediators, $p67^{phox}$ will be translocated to the membrane to form an active enzyme complex with flavocytochrome b_{558} to regulate electron transfer from NADPH to flavin adenine dinucleotide. Once activated, superoxide is released into the phagocytic vacuole or into the extracellular space (Mizuki et al., 1998; Han et al., 1998; Nisimoto et al., 1999). The upregulation of these five genes induced by *E. ictaluri* vaccination suggests that they might play important roles in the immune defense system in channel catfish in response to *E. ictaluri* vaccination and/or infection.

In summary, 57 different genes were isolated from *E. ictaluri* vaccinated *vs.* non-vaccinated channel catfish anterior kidney. Of the 57 ESTs, 43 were induced at least 2-fold higher in all three vaccinated fish compared to that in unvaccinated control fish. Of the 43 upregulated genes, five were consistently upregulated greater than 10-fold, including two highly upregulated (>20-fold) glycosyltransferase and Toll-like receptor 5. The transcriptional levels of GTPase 1, coatomer protein complex zeta 1, and type II arginine deiminase were consistently induced greater than 10-fold. MHC class I α chain and transposase were upregulated greater than 10-fold in two of the three vaccinated fish. The 43 upregulated genes also included 19 moderately upregulated (3–10-fold) and 17 slightly upregulated (2–3-fold). Our results suggest that subtractive cDNA hybridization and QPCR are powerful cost-effective techniques to identify differentially expressed genes in response to bacteria challenge. Significant upregulation of multiple genes induced by modified live *E. ictaluri* vaccination suggests that they might play important roles in the host defense by priming the immune system for response to *E. ictaluri* vaccination and/or infection.

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